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dad-1, an endogenous programmed cell death suppressor in *Caenorhabditis elegans* and vertebrates

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Programmed cell death (apoptosis) is a normally occurring process used to eliminate unnecessary or potentially harmful cells in multicellular organisms. Recent studies demonstrate that the molecular control of this process is conserved phylogenetically in animals. The *dad-1* gene, which encodes a novel 113 amino acid protein, was originally identified in a mutant hamster cell line (tsBN7) that undergoes apoptosis at restrictive temperature. We have identified a *dad-1* homologue in *Caenorhabditis elegans* (*Ce-dad-1*) whose predicted product is >60% identical to vertebrate DAD-1. A search of the sequence databases indicated that DAD-1-like proteins are also expressed in two plant species. Expression of either human *dad-1* or *Ce-dad-1* under control of a *C.elegans* heat-shock-inducible promoter resulted in a reduction in the number of programmed cell death corpses visible in *C.elegans* embryos. Extra surviving cells were present in these animals, indicating that both the human and *C.elegans dad-1* genes can suppress developmentally programmed cell death. *Ce-dad-1* was found to rescue mutant tsBN7 hamster cells from apoptotic death as efficiently as the vertebrate genes. These results suggest that *dad-1*, which is necessary for cell survival in a mammalian cell line, is sufficient to suppress some programmed cell death in *C.elegans*.

Keywords: apoptosis/*Caenorhabditis elegans*/*dad-1*/plants/programmed cell death

Introduction

Multicellular organisms are capable of carrying out a program of cell suicide that eliminates unnecessary or harmful cells during development and maintenance of tissues, and under pathological conditions (reviewed in Ellis *et al.*, 1991; Raff, 1992; Thompson, 1995). Most programmed cell death is called apoptosis; this type of cell death is typically accompanied by a stereotyped repertoire of morphological and biochemical changes that occur irrespective of species or cell type. These events include plasma membrane blebbing, condensation of cytoplasm, compaction of chromatin, and degradation of nuclear DNA into oligonucleosome-size fragments (Wyllie *et al.*, 1980).

Regulatory mechanisms exist to ensure that this genetically programmed process of cell death is not activated inappropriately. It has been proposed that, at least in vertebrates, all cells may be poised to undergo programmed death and must receive signals constantly to repress this programme, thereby allowing their survival (Raff, 1992). The molecular mechanisms that control programmed cell death appear to be conserved throughout the animal kingdom (reviewed in Hengartner, 1994; Steller, 1995). The mammalian cell death suppressor gene, *bcl-2*, blocks developmentally programmed cell death when expressed in the nematode *Caenorhabditis elegans* (Vaux *et al.*, 1992). Moreover, the *ced-9* gene of *C.elegans*, which protects cells from undergoing programmed death during development of the nematode (Hengartner *et al.*, 1992), is a member of the *bcl-2* family (Hengartner and Horvitz, 1994). A gene required for execution of programmed cell death also appears to be conserved: *ced-3*, which is required for cell death in *C.elegans*, encodes a protein that is similar to a cysteine protease, interleukin-1β-converting enzyme (ICE) (Yuan *et al.*, 1993). CED-3, ICE and Nedd2/Ich-1, another member of the ICE-like cysteine protease family, can all induce apoptosis when expressed in mammalian cells (Miura *et al.*, 1993; Kumar *et al.*, 1994; Wang *et al.*, 1994).

A candidate for a new endogenous programmed cell death suppressor gene was recently identified from studies of a temperature-sensitive mutant hamster cell line, tsBN7, that undergoes apoptosis when incubated at restrictive temperature (Nakashima *et al.*, 1993). This cell line was found to carry a mutation in the *dad-1* (*d*efender against *a*poptotic *d*eath) gene. The mutant *dad-1* product, which contains a single amino acid substitution, is not detectable in tsBN7 cells following a shift to restrictive temperature (Nakashima *et al.*, 1993). This loss of DAD-1 precedes the onset of apoptosis, suggesting that it may be the event that triggers programmed cell death. The apoptotic death of tsBN7 cells can be rescued with a wild-type copy of the *dad-1* gene. *dad-1* encodes a novel hydrophobic protein of 113 amino acids which is well conserved among vertebrates: humans and hamsters express identical DAD-1 proteins, which are 91% identical to DAD-1 in frogs (Nakashima *et al.*, 1993).

Here we describe the isolation of a *C.elegans dad-1* homologue (*Ce-dad-1*) and demonstrate that expression of human and *C.elegans dad-1* genes is sufficient to inhibit developmentally programmed cell death in *C.elegans*. In addition, we show that *Ce-dad-1* can substitute for hamster *dad-1* in cultured cells. These results indicate that *dad-1* is an evolutionarily conserved inhibitor of programmed cell death.

Results

Human dad-1 inhibits developmentally programmed cell death in C.elegans

The observation that tsBN7 cells undergo apoptosis at restrictive temperature suggests the possibility that wild-type *dad-1* may normally inhibit programmed cell death. Alternatively, *dad-1* might perform a more general housekeeping function required for cell viability which, when eliminated, results in apoptosis. We sought to test these alternatives by asking whether human *dad-1* can interfere with programmed cell death in *C.elegans*. Since virtually all programmed cell deaths in the nematode are controlled by a single genetic pathway (Ellis *et al.*, 1991) which is conserved phylogenetically (Vaux *et al.*, 1992; Miura *et al.*, 1993; Yuan *et al.*, 1993; Hengartner and Horvitz, 1994), *C.elegans* is a useful system in which to test the activity of putative cell death regulators from other systems (e.g. Vaux *et al.*, 1992; Sugimoto *et al.*, 1994). The reproducibility in the pattern of dying cells in this animal provides a sensitive assay for alterations in the occurrence of programmed cell death: of the 1090 somatic cells produced during the development of a *C.elegans* hermaphrodite, 131 die at precise times and locations, which are predicted from the known cell lineage of the animal (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). The cell corpses, which are eventually engulfed by neighboring cells, are easily scored in living animals using light microscopy.

To test the effect of human *dad-1* on programmed cell death in *C.elegans*, we generated transgenic nematodes carrying a human *dad-1* cDNA under the control of a *C.elegans* heat-shock promoter, *hsp16-2* (Jones *et al.*, 1986). This construct (*hs-hu-dad-1*) was microinjected along with a plasmid carrying a phenotypic marker gene, *rol-6(su1006)* (Mello *et al.*, 1991), into the gonads of *ced-1* mutant adults. In *ced-1* animals, cell corpses persist because of a defect in cell corpse engulfment (Hedgecock *et al.*, 1983); this phenotype facilitates the analysis of the cell death phenotype. Transgenic worms were selected by scoring for the Rol phenotype.

To assess the effect of *dad-1* on programmed cell death, we first asked whether expression of *dad-1* affected the number of cell corpses seen at a particular stage in embryonic development. A heat-shock was given during early gastrulation (100–200 min after first cleavage), well before the onset of the first programmed cell death (~230 min). The number of cell corpses was scored at 'comma' stage (~380 min), by which time ~60 cell deaths have occurred in wild-type embryos. Cell corpses were scored in transgenic and control embryos both with and without a prior heat-shock. Without a heat-shock, the number of corpses seen in transgenic embryos (14.8 ± 3.0 corpses; expressed as mean ± s.d.) was not significantly different ($P > 0.2$) from that seen in control *ced-1* embryos (14.6 ± 3.0, non-heat-shocked; 15.4 ± 2.9, heat-shocked) (Figure 1A). In contrast, after a heat-shock, progeny from transgenic animals carrying *hu-dad-1* showed significantly fewer corpses (8.7 ± 2.8; $P < 0.00002$; Figure 1B). A second independent transgenic line showed a similar effect (data not shown). Thus, expression of *hu-dad-1* reduces the number of cell corpses in developing *C.elegans* embryos.

To test whether the heat-shock-dependent reduction in cell corpse number might result from a general delay in differentiation, we compared the development of heat-shocked and non-heat-shocked embryos from transgenic animals. Animals from both populations developed at the same rate based on three criteria: differentiation of gut cells observed by Nomarski microscopy, production of gut granules (a gut-specific marker), and duration of time required to reach the comma stage. All three events occur before or at the same time in development that the cell corpse assay was performed. These results indicated that there was no obvious delay in differentiation in the heat-shocked transgenic animals.

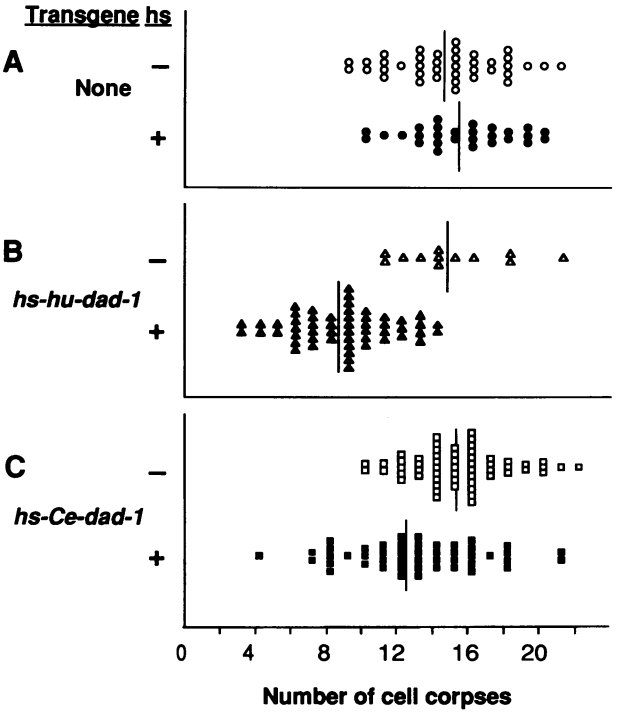

Fig. 1. Distribution of cell corpse number in embryos. The total number of cell corpses seen in embryos at the 'comma stage' (380 min after the first cell division) is shown. Each symbol represents a single embryo. Embryos were scored for number of cell corpses following a heat-shock (closed symbols) or with no heat-shock (open symbols). (**A**) *ced-1* control strain. (**B**) *hs-hu-dad-1* transgenic strain (*ced-1; wEx58*). (**C**) *hs-Ce-dad-1* transgenic strain (*ced-1; wIs14*). Vertical lines indicate the mean number of corpses. A reduction in cell corpse number following heat-shock was observed in at least two independent transgenic lines for both *hu-dad-1* and *Ce-dad-1* (data not shown). Neither the presence of the marker plasmid pRF4 nor that of the *hsp16-2* vector appear to affect programmed death (data not shown; Hengartner and Horvitz, 1994; Sugimoto *et al.*, 1994).

The reduction in number of visible cell corpses might be caused by suppression of programmed cell death or enhanced engulfment of cell corpses. To test these alternatives, we assayed for the presence of extra surviving cells in fully developed animals well after the time of heat-shock. The presence of additional cells above that seen in wild-type animals would imply that some cells have escaped programmed death. We focused on the cells of the anterior portion of the feeding organ, or pharynx, since the nuclei of all these cells are easily scored (Ellis and Horvitz, 1991; Hengartner and Horvitz, 1994). Embryos from the *hs-hu-dad-1* transgenic line were given a heat-shock, and nuclei in the anterior pharynx were counted. The difficulty of accurately scoring pharynx

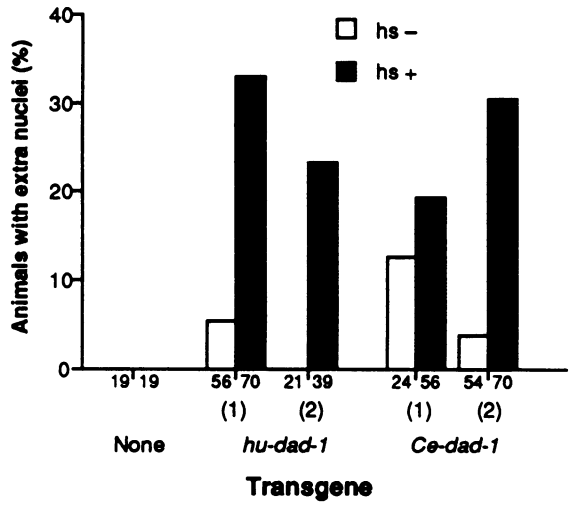

Fig. 2. Extra anterior pharynx nuclei. The number of nuclei in the anterior pharynx (Ellis and Horvitz, 1991) was scored at the L4 stage. The fraction of animals with extra nuclei is shown. Numbers below each bar indicate the number of animals scored. For each of the indicated constructs, two independent strains were examined: *hs-hu-dad-1* integrant # 1 is *wIs7*, *hs-hu-dad-1* integrant # 2 is *wIs8*, *hs-Ce-dad-1* integrant # 1 is *wIs10*, and *hs-Ce-dad-1* integrant # 2 is *wIs14*. Wild-type animals undergo 14 programmed cell deaths and produce 49 surviving nuclei in this region during embryonic development. There was no obvious preference for survival of certain cells over others, suggesting that *dad-1* does not act in a cell-type-specific manner. In some of the transgenic lines carrying *dad-1*, a significant number of surviving cells was seen, even without heat-shock. This was never observed with the control strain and presumably results from basal expression from the heat-shock promoter.

nuclei in embryos and early larvae necessitated scoring of nuclei in the pharynx in late larvae, ~48 h after the heat-shock was given. In control animals lacking a transgene, no animals had extra nuclei irrespective of heat-shock. In contrast, many of the heat-shocked transgenic animals (line 1, 33%; line 2, 23%; Figure 2) had extra nuclei in the anterior pharynx. Up to three extra nuclei were observed in this region, in which 14 cells normally undergo programmed death. The positions of the extra nuclei were consistent with the positions of extra nuclei that appear as the result of a block in programmed cell death (e.g. in *ced-3* mutants), suggesting that these nuclei indeed correspond to cells that normally undergo programmed cell death. These results demonstrate that expression of human *dad-1* promotes the survival of cells normally programmed to die during embryogenesis.

Isolation of a C.elegans dad-1 homologue

The above results suggest that *dad-1* performs a cell death-inhibiting function which may be evolutionarily conserved. To determine whether *C.elegans* expresses an endogenous *dad-1* gene, we sought to identify a *dad-1* homologue in *C.elegans* (*Ce-dad-1*). From a computer-based homology search, we identified an existing *C.elegans* expressed sequence tag (EST), wEST02556, that encodes a product with high sequence similarity to vertebrate *dad-1* gene products. Since this cDNA clone appeared to be partial, we used the insert of wEST02556 to isolate additional cDNAs containing the complete *dad-1* open reading frame. From a screen of 75 000 clones, eight positive cDNA clones were identified. The two longest inserts (510 and 540 bases) were sequenced (Figure 3A) and the sequences were found to be identical to each other with the exception of the length of the poly(A) tail. The nine nucleotides at the 5′-end of these cDNAs were identical to the last nine bases of the 22 nucleotide leader sequence, SL1, which is *trans*-spliced to the 5′ termini of ~60% of all *C.elegans* mRNAs (Krause and Hirsh, 1987). A *trans*-splicing acceptor consensus sequence (Emmons, 1988; Conrad *et al.*, 1991) is present at the site in the *C.elegans* genomic sequence corresponding to the presumptive splice junction (not shown). Thus, the cDNAs appear to be almost complete and include the entire protein-coding region.

The sequence of the *Ce-dad-1* cDNAs predicts an open reading frame containing 113 amino acids, the same length as the vertebrate DAD-1 proteins (Figure 3B). The predicted *C.elegans* product is 61% identical and 81% similar to human and hamster DAD-1. The structural conservation is strongest following the N-terminal 23 amino acids of the protein. The glycine at position 38, which is altered to arginine in the tsBN7 cell line (Nakashima *et al.*, 1993), is conserved in all known DAD-1 proteins in animals. In addition, the position of the single intron–exon junction in the coding region at codon 71 is conserved between hamsters and *C.elegans* (not shown).

From the sequence databases, we also identified sequences from two plant species, *Arabidopsis thaliana* and the rice *Oryza sativa*, that encode products with high sequence similarity to the animal DAD-1 products. Although these both appear to be partial sequences, the predicted *Arabidopsis* polypeptide has an extension at the N-terminus compared with the animal sequences. The apparently incomplete sequence of the predicted rice polypeptide showed similarity to the C-terminal 66 amino acids of the animal DAD-1 proteins. The plant sequences are >40% identical to *Ce*-DAD-1 and are >80% identical to each other (Figure 3B). Thus, *dad-1* appears to be well conserved across two kingdoms.

Overexpression of Ce-dad-1 inhibits developmentally programmed cell death in C.elegans

To test whether *Ce-dad-1*, like the mammalian gene, can act as a suppressor of programmed cell death, we examined the effect of overexpressing it in *C.elegans*. We placed the *Ce-dad-1* cDNA under the control of the *hsp16-2* promoter and constructed transgenic worms as described earlier for the *hu-dad-1* transgenic strains. As seen with the human gene, *Ce-dad-1* reduced the number of cell corpses in a heat-shock-dependent manner (Figure 1C): heat-shocked *hs-Ce-dad-1* transgenic embryos had 12.4 ± 3.7 corpses, compared with 15.2 ± 2.7 for the non-heat-shocked animals, a statistically significant difference ($P < 0.00002$). In addition, we analyzed for the presence of extra nuclei in pharynxes in the *Ce-dad-1* transgenic lines. Up to three extra surviving nuclei were observed in the anterior pharynx of heat-shocked *hs-Ce-dad-1* animals (line 1, 19%; line 2, 30%; Figure 2). These results demonstrate that *Ce-dad-1* can inhibit developmentally programmed cell deaths.

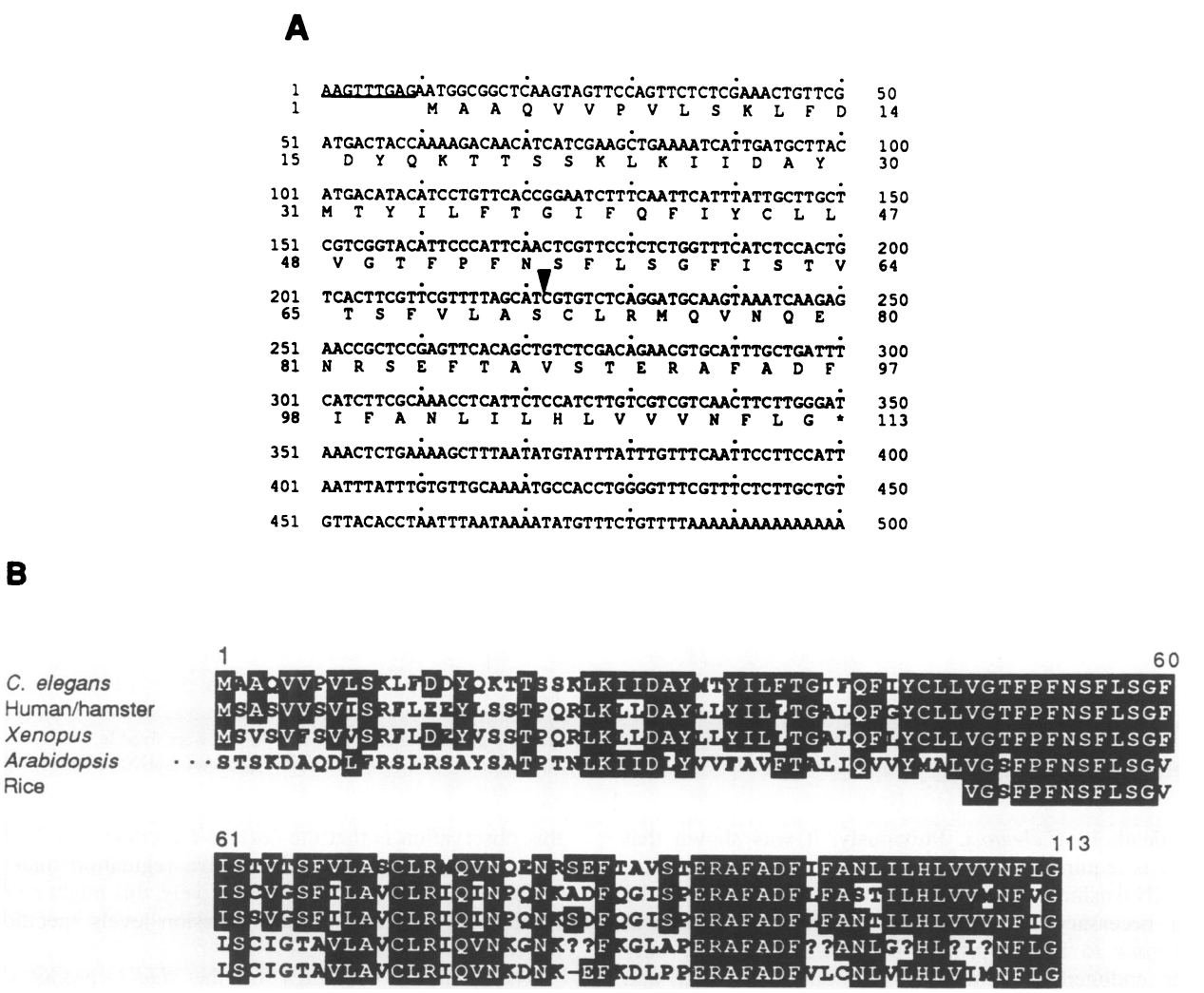

Fig. 3. (**A**) Sequence of a *Ce-dad-1* cDNA and its predicted peptide. cDNA clones 1a and 2a were identical in sequence with the exception of the length of the poly(A) tail. The partial (9 nt) SL1 *trans*-spliced leader sequence is underlined (Krause and Hirsh, 1987). A splicing junction is shown by an arrowhead. EMBL accession number for the *Ce-dad-1* cDNA sequence: X89080. (**B**) Predicted amino acid sequence of *Ce*-DAD-1 and DAD-1 proteins from other species. Residues that are identical in *Ce-dad-1* and one or more of the other four proteins are given as white-on-black letters. A hyphen indicates a single-residue gap introduced to maximize the alignment. Sequence ambiguities in the *Arabidopsis* EST (expressed sequence tag) are shown as question marks. Dots indicate that longer polypeptides are predicted. The *Arabidopsis* EST sequence encodes a longer polypeptide; only the C-terminal 113 amino acids are shown. The *Arabidopsis* and animal sequences diverge at their N termini. The rice EST is probably partial and all deduced amino acids from the available sequence are shown.

Ce-dad-1 complements hamster dad-1^{ts} mutant cells

Mammalian and *C.elegans* DAD-1 proteins are structurally similar and both possess comparable programmed cell death-suppressing activity when expressed in *C.elegans*. To test whether *Ce-dad-1* is functionally interchangeable with mammalian *dad-1*, we examined the ability of *Ce-dad-1* to complement the hamster tsBN7 mutant cells. The *C.elegans*, human and *Xenopus dad-1* cDNAs placed under the control of the SRα promoter (Takebe *et al.*, 1988) were introduced into tsBN7 cells and the resulting transformants tested for temperature-sensitive lethality. *Ce-dad-1* rescued the temperature-sensitivity of the hamster line as efficiently as the human and *Xenopus dad-1* genes (Table I, Figure 4). Since the hamster DAD-1 protein diminishes to undetectable levels in tsBN7 cells following a shift to restrictive temperature (Nakashima *et al.*, 1993), these results strongly suggest that *Ce-dad-1* can fully substitute for mammalian *dad-1* in hamster cells.

Table I. Complementation of tsBN7 cells by *Ce-dad-1*

Plasmids	No. of colonies (per 2×10^5 cells)	
	39.5°C[a]	33.5°C + G418[b]
pcDSRα-Ce-dad-1	304	166
pcDSRα-hu-dad-1	294	186
pcDSRα-Xe-dad-1	180	152
no DNA	0	n.t.[c]

[a]Cells were incubated at 33.5°C for 2 days and then at 39.5°C (a non-permissive temperature for tsBN7) to test the rescue of temperature-sensitivity.
[b]33.5°C (a permissive temperature for these cells) in the presence of G418 (400 μg/ml) to test for transformation efficiency.
[c]Not tested.

Discussion

We have shown that expression of both the human and *C.elegans dad-1* genes is sufficient to inhibit programmed

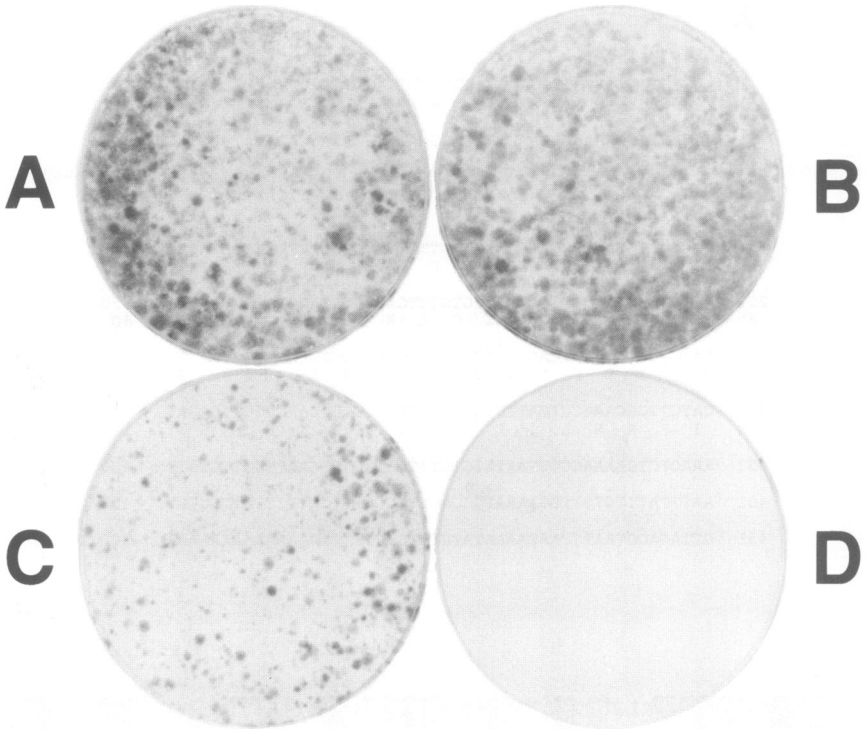

Fig. 4. Complementation of the temperature-sensitive lethality of tsBN7 cells by *dad-1* genes. Colonies grown at 39.5°C were fixed in 10% formaldehyde and stained with 0.3% crystal violet. (**A**) Human *dad-1* cDNA. (**B**) *C.elegans dad-1* cDNA. (**C**) *Xenopus dad-1* cDNA. (**D**) No DNA.

cell death in *C.elegans*. Previously, it was shown that *dad-1* is required to prevent apoptosis of cultured hamster cells (Nakashima *et al.*, 1993). Thus, *dad-1* appears to be both necessary in mammalian cells and sufficient in *C.elegans* to inhibit programmed cell death. The only other endogenous genes that are both necessary and sufficient to block cell death are certain members of the *bcl-2* gene family.

The cell death-suppressing activity of human and *C.elegans dad-1* that we observed was somewhat weaker than was seen in similar experiments using human *bcl-2* (Vaux *et al.*, 1992), *C.elegans ced-9* (Hengartner and Horvitz, 1994) or baculovirus *p35* (Sugimoto *et al.*, 1994). A possible explanation for the relatively weaker effect of DAD-1 is that it may be required continuously in cells to promote cell survival (as is suggested by the behavior of tsBN7 cells). If endogenous DAD-1 is normally eliminated in cells programmed to die, then transient expression of *dad-1* from the heat-shock promoter might rescue these cells only as long as DAD-1 is present. DAD-1 produced during the heat-shock might later diminish to levels that are insufficient to sustain cell survival, thus resulting in the delayed death of some cells. (Surviving pharynx nuclei were scored more than 48 h after heat-shock.) Alternatively, the *hsp16-2* promoter used in these studies may provide insufficient levels of DAD-1 to inhibit programmed cell death efficiently. For example, cells programmed to die might negatively regulate endogenous DAD-1 activity and high levels of DAD-1 might be required to overcome this negative regulation.

The *hu-dad-1* transgene appears to suppress programmed cell death in *C.elegans* somewhat more efficiently than does the endogenous *Ce-dad-1* expressed as a transgene (Figure 1). One possible explanation for this observation is that the *hu-dad-1* product may be less sensitive to some form of negative regulation than the endogenous gene product. Alternatively, this might merely result from a difference in expression levels specific to the transgenic lines.

The primary structures of the *dad-1* products in *C.elegans* and mammals are highly similar (61% identity between *C.elegans* and humans). However, we have been unable to recognize conserved structural motifs that might shed light on the biochemical function of DAD-1. The DAD-1 polypeptides are highly hydrophobic, suggesting that they are likely to reside in a membrane; preliminary biochemical experiments confirm that DAD-1 is probably an intrinsic membrane protein (T.Nishimoto, unpublished data). The high degree of structural conservation between DAD-1 proteins is likely to reflect a similarity in function, since *Ce-dad-1* can substitute for hamster *dad-1*.

We identified two plant genes in the sequence databases that are similar to *dad-1*. Given the apparent role of DAD-1 proteins in suppression of programmed cell death in animals, it is conceivable that the plant genes might perform a similar function. Programmed cell death is observed in plants under various conditions such as senescence, during the formation of the vascular tissue known as xylem (Chasan, 1994), sex determination (DeLong *et al.*, 1993), and the hypersensitive response to pathogens (Dietrich *et al.*, 1994; Greenberg *et al.*, 1994). However, it is as yet unknown whether any of these examples of programmed cell death in plants is related to programmed cell death in animals.

Although divergent stimuli, such as growth factor withdrawal, glucocorticoids, heat-shock, viral infection, presence of free radicals, and ultraviolet or gamma radiation (reviewed in Thompson, 1995), can induce pro-

grammed cell death, execution of the cell death programme appears to depend on a common molecular pathway. This notion is supported by studies showing that a number of cell death suppressors can prevent cell death induced by various stimuli and in different organisms. For example, *bcl-2* has been shown to inhibit cell death in various cell types (Vaux *et al.*, 1988; Strasser *et al.*, 1991; Garcia *et al.*, 1992), and in widely divergent species (Vaux *et al.*, 1992). Bcl-x_L, a Bcl-2-like protein from chicken, can also suppress apoptosis of mammalian cells (Boise *et al.*, 1993), and a number of viruses also express Bcl-2-like cell death suppressors [e.g. adenovirus E1B 19k (Rao *et al.*, 1992), Epstein–Barr virus BHRF1 (Henderson *et al.*, 1993) and African swine fever virus LMW5-HL (Neilan *et al.*, 1993)]. In addition, some cell death suppressors have been identified that appear to be unrelated to Bcl-2. For example, baculovirus p35 is a novel protein that can inhibit programmed cell death in a diversity of invertebrate and vertebrate systems (Clem *et al.*, 1991; Rabizadeh *et al.*, 1993; Hay *et al.*, 1994; Sugimoto *et al.*, 1994). Another cell death suppressor from baculovirus, IAP, has a putative zinc-finger domain and can inhibit apoptosis in insect cells brought on by different stimuli (Crook *et al.*, 1993; Birnbaum *et al.*, 1994; Clem and Miller, 1994). DAD-1 has no sequence similarity to any of these cell death suppressor genes.

It is not known how the mechanisms of programmed cell death suppression by *dad-1* and *bcl-2* are related. Unlike the apoptotic suppressor, LMP1, encoded by the Epstein–Barr virus (Henderson *et al.*, 1991), *dad-1* does not appear to exert its cell death-suppressing function by promoting expression of *bcl-2*, since apoptosis of tsBN7 cells is not blocked by expression of *bcl-2* in these cells (Nakashima *et al.*, 1993). Furthermore, unlike Bcl-2, which is expressed in a tissue- and stage-specific manner (Hockenbery *et al.*, 1991; Merry *et al.*, 1994), *dad-1* is transcribed at relatively high levels in all human tissues examined (Nakashima *et al.*, 1993).

It will be of interest to determine the relationship between *dad-1* and components that promote programmed cell death. CED-3, a presumptive cysteine protease, is required for all developmentally programmed cell deaths in *C.elegans* (Ellis and Horvitz, 1986), and expression of CED-3 and CED-3-related proteins, including mammalian ICE and Nedd2/Ich-1, induces apoptosis in cultured mammalian cells (Miura *et al.*, 1993; Kumar *et al.*, 1994; Wang *et al.*, 1994). It is possible that DAD-1 directly or indirectly inhibits CED-3/ICE protease activity. An example of an apoptotic inhibitor that acts by such a mechanism is the cowpox virus *crmA* gene product. This protein is a potent inhibitor of ICE protease activity (Ray *et al.*, 1992) and suppresses programmed cell death (Miura *et al.*, 1993; Gagliardini *et al.*, 1994). Alternatively, DAD-1 may be a cell survival factor, and cleavage of DAD-1 by a CED-3/ICE-like protease might trigger programmed cell death. Consistent with the view that elimination of DAD-1 might result in programmed cell death is the observation that mutant *dad-1* product in tsBN7 cells rapidly decays to undetectable levels after a shift to restrictive temperature; apoptosis follows shortly thereafter. ICE is a cysteine protease that cleaves at some Asp-X bonds (X is any amino acid) (Thornberry *et al.*, 1992) and several substrates for ICE-like proteases have been identified (Kaufmann *et al.*, 1993; Casciola-Rosen *et al.*, 1994; Lazebnik *et al.*, 1994). However, it is not clear whether cleavage of these substrates is relevant for the induction of programmed cell death. Interestingly, aspartic acid residues at positions 28 and 96 are conserved in all DAD-1 proteins (Figure 3B) and it would be of interest to determine whether DAD-1 is cleaved by ICE-like proteases at either of these sites.

Although *dad-1* possesses cell death-inhibiting activity, it is possible that it also performs other functions. Both animals and higher plants express DAD-1-like proteins, implying that the *dad-1* gene originally arose in a unicellular organism, before the plant and animal kingdoms diverged. The ubiquitous expression of *dad-1* (Nakashima *et al.*, 1993) and its conservation across two kingdoms might indicate that *dad-1* plays a role that is vital for the function of all cells. The function of *dad-1* and its relationship with other components that participate in programmed cell death may be further elucidated once mutations that eliminate *dad-1* function in *C.elegans* have been identified.

Materials and methods

C.elegans strain and general methods

Nematodes were grown on agar plates seeded with *Escherichia coli* strain OP50 as described (Brenner, 1974). Unless otherwise indicated, strains were grown at 20°C. To facilitate scoring cell corpses, an engulfment-defective mutant, *ced-1(e1735)* I (Hedgecock *et al.*, 1983), was used.

Isolation of Ce-dad-1

C.elegans expressed sequence tags (ESTs) in the GenBank database were analyzed for sequence similarity to the human *dad-1* cDNA, using the TFASTA program of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI). The single EST, wEST02556, showed significant similarity to the human *dad-1* gene.

A *C.elegans* cDNA library (Barstead and Waterston, 1989) constructed in the lambda ZAP vector (Stratagene) was screened for additional *Ce-dad-1* cDNA clones using the entire insert (*Xho*I–*Eco*RI 320 bp fragment) of CEESW25, the plasmid corresponding to wEST02556, as a probe. Eight positive clones were obtained from 75 000 clones. The inserts from the positive lambda clones were recovered by excision in the pBluescript SK–vector by co-infection with a helper phage (Short *et al.*, 1988). The two longest inserts (1a, 510 bases and 2a, 540 bases) were sequenced using the Sequenase Version 2.0 Kit (USB), and the nucleotide sequences of both were found to be identical, with the exception of the length of the poly(A) tail. As described in the text, these appear to be represent the entire Ce-dad-1 coding region.

Database search for dad-1 homologues

A homology search of the sequence databases was performed with the TFASTA program of the UWGCG package and the BLAST Network Service at the National Center for Biotechnology Information (Bethesda, MD), using the Ce-DAD-1 polypeptide sequence as a query. The *A.thaliana* cDNA (127H23T7) was sequenced by T.Newman, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI. The rice cDNA (R1413_1A) was sequenced by Y.Minobe, Dept. Rice Genome Research Program, National Institute of Agrobiological Resources, Tsukuba, Ibaraki, Japan. The deduced *Arabidopsis* protein has additional sequences at the N-terminus compared with the animal proteins. The rice cDNA sequence is likely to be partial and presumably lacks sequences corresponding to the N-terminus of rice DAD-1.

Strain construction

hu-dad-1 transgenic strains. To construct transgenic lines, plasmid pRRH103 was microinjected along with a phenotypic marker plasmid, pRF4 (Mello *et al.*, 1991), into the germline of adult *ced-1(e1735)* hermaphrodites. pRRH103 was constructed by inserting a 0.53 kb *Hin*cII–*Xba*I *hu-dad-1* cDNA fragment (Nakashima *et al.*, 1993) into the *Nhe*I site of pPD49.78 (provided by A.Fire) such that the *hu-dad-1*

cDNA is expressed under the control of *hsp16-2* promoter. pRRH103 was injected at a concentration of 150 μg/ml and pRF4 at 100 μg/ml. Co-injected plasmids are believed to assemble into an extrachromosomal array that segregates in a non-Mendelian fashion (Mello *et al.*, 1991). Animals carrying the arrays were detected by observing the phenotype of late larvae and adults: transgenic animals generally exhibit a rolling (Rol) phenotype as a result of expression of *rol-6(su1006)* from the marker plasmid pRF4. The obtained transgenic line, *wEx58*, segregates ~60% Rol animals. To integrate the transgene into the genome, *ced-1* transformants carrying extrachromosomal array *wEx58* were irradiated with gamma-rays (3500 rads) and integrants (*ced-1*; *wIs8* and *ced-1; wIs9*) in which the transgene was stably transmitted were identified.

Ce-dad-1 transgenic strains. Transgenic strains were constructed as described above with plasmids pAS78(dad-1)-1 and pRF4. pAS78(dad-1)-1 was injected at a concentration of 20 μg/ml and pRF4 at 180 μg/ml. pAS78(dad-1)-1 was constructed by inserting the 510 bp *Xba*I–*Kpn*I fragment from the cDNA clone 1a into the vector plasmid pPD49.78 that had been digested with *Nhe*I and *Kpn*I such that *Ce-dad-1* cDNA is expressed under the control of the *hsp16-2* promoter. A transformant, *ced-1; wEx23*, was gamma-irradiated to obtain the integrants *ced-1; wIs10* and *ced-1; wIs14*.

The integrated constructs were back-crossed at least four times to the original *ced-1* strain, and strains homozygous for each integrant were used.

Assaying for cell corpse number

The method described by Sugimoto *et al.* (1994) was used with slight modification to quantitate cell corpses. For heat-shock experiments, gravid Rol hermaphrodite adults were cut open and embryos were collected and given a heat-shock at 33°C for 30 min. After 3.5–5.5 h, embryos at the comma stage (Sulston *et al.*, 1983) were collected and the number of cell corpses scored by Nomarski microscopy. Using the known developmental time course of wild-type embryos, we estimated these embryos received the heat-shock between 100 and 200 min after first cleavage. The comma stage was chosen for scoring because it allows precise staging of embryos. This stage also facilitates scoring because of the absence of active movement, which begins shortly after this stage. Because the engulfment defect in the *ced-1* mutant is incomplete, most corpses are eventually engulfed. Therefore the number of corpses observed is always substantially less than the number of deaths that have occurred.

Scoring surviving nuclei in the pharynx

Embryos were heat-shocked as described above. Approximately 2 days after heat-shock, L3 or L4 larvae were anaesthetized by mounting them on an agar pad containing 10 mM NaN_3. The numbers of extra nuclei in the anterior pharynx were then counted using Nomarski microscopy. Some experiments were done blind, with the observer unaware of whether the worms had been heat-shocked at the time of scoring. The results from both blind and non-blind experiments were similar; thus Table I shows the combined data from all experiments.

Complementation of tsBN7

pcDSRα296 (Takebe *et al.*, 1988) was used as an expression vector for *dad-1* cDNAs. pcDSRα-hu-dad-1 is described by Nakashima *et al.* (1993). pcDSRα-Xe-dad-1 and pcDSRα-Ce-dad-1 were constructed by inserting a *Xe-dad-1* or *Ce-dad-1* cDNA fragment into the *Eco*RI site of pcDSRα296. *dad-1* expression vectors were co-transfected with pSV2-neo (a marker for transformation efficiency) into tsBN7 cells (2×10^5 cells/100 mm dish) by the calcium phosphate precipitation method (Chen and Okayama, 1987). The cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 10% CO_2. Following transfection, cultures were incubated at 33.5°C for 2 days and then at 39.5°C (a non-permissive temperature for tsBN7) to test for rescue of temperature sensitivity, or at 33.5°C (a permissive temperature for these cells) in the presence of G418 (400 μg/ml) to test for transformation efficiency. The plasmid vector alone does not rescue tsBN7 (Nakashima *et al.*, 1993).

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